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Substrate induction and glucose repression of maltose utilization by *Streptomyces coelicolor* A3(2) is controlled by *malR*, a member of the *lacI–galR* family of regulatory genes

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Summary

***malR* of *Streptomyces coelicolor* A3(2) encodes a homologue of the LacI/GalR family of repressor proteins, and is divergently transcribed from the *malEFG* gene cluster, which encodes components of an ATP-dependent transport system that is required for maltose utilization. Transcription of *malE* was induced by maltose and repressed by glucose. Disruption or deletion of *malR* resulted in constitutive, glucose-insensitive *malE* transcription at a level markedly above that observed in the parental *malR*$^+$ strain, and overproduction of MalR prevented growth on maltose as carbon source. Consequently, MalR plays a crucial role in both substrate induction and glucose repression of maltose utilization. *malR* is expressed from a single promoter with transcription initiating at the first G of the predicted GTG translation start codon.**

Introduction

Members of the genus *Streptomyces* are Gram-positive, mycelial soil bacteria with a high genomic G+C content, and undergo a complex process of morphological development that normally results in sporulation (Chater and Losick, 1996). They also produce a wide variety of secondary metabolites, many of which are used as antibiotics in human medicine and agriculture (Miyadoh, 1993). Streptomycetes are saprophytes, obtaining nutrients and energy by solubilizing organic material in the soil through the production of extracellular hydrolytic enzymes that include amylases, xylanases, cellulases and proteases (McCarthy and Williams, 1992). Alpha-amylase genes (*aml*) have been cloned from a variety of streptomycetes, and the regulation of α-amylase production has been studied in *Streptomyces limosus* (Long *et al*., 1987; Virolle *et al*., 1988), *Streptomyces venezuelae* (Virolle and Bibb, 1988), *Streptomyces thermoviolaceus* (Bahri and Ward, 1990), *Streptomyces hygroscopicus* (Graefe *et al*., 1986) and *Streptomyces kanamyceticus* (Flores *et al*., 1993). Transcription of at least some of these genes is induced by starch-derived di- and oligosaccharides, such as maltose and maltotriose, and *aml* genes probably belong to the maltose regulons of most, if not all, streptomycetes. While induction of *aml* transcription in *S. venezuelae* is repressed by glucose, it is not repressed by this sugar in *S. limosus* (Virolle *et al*., 1988) and *S. thermoviolaceus* (Bahri and Ward, 1990); in both of the latter strains, mannitol acts as a repressing carbon source. However, when cloned in *Streptomyces coelicolor* A3(2) or in *Streptomyces lividans*, *aml* of *S. limosus* adopts the regulatory characteristics of its surrogate host, with induction of *aml* transcription being repressed by glucose and not by mannitol (Virolle *et al*., 1988).

The mechanism of glucose repression in streptomycetes is not understood. While phosphoenolpyruvate (PEP)-dependent fructose phosphotransferase systems occur in some streptomycetes (Titgemeyer *et al*., 1995), attempts to identify PEP-dependent glucose phosphotransferase systems in several *Streptomyces* species have failed (Sabater *et al*., 1972; Novotná and Hostálek, 1985). This, and the absence of fluctuations in cAMP levels with changes in carbon source in *S. coelicolor* (Hodgson, 1980) and in *S. venezuelae* (Chatterjee and Vining, 1982), suggest that the mechanism of glucose repression is markedly different from that in *Escherichia coli* (Postma *et al*., 1993; 1996). In other Gram-positive bacteria with genomic DNA of lower G+C content, such as *Bacillus subtilis*, *Bacillus megaterium* and *Staphylococcus xylosus*, CcpA, a homologue of the LacI–GalR family of regulatory proteins, acts

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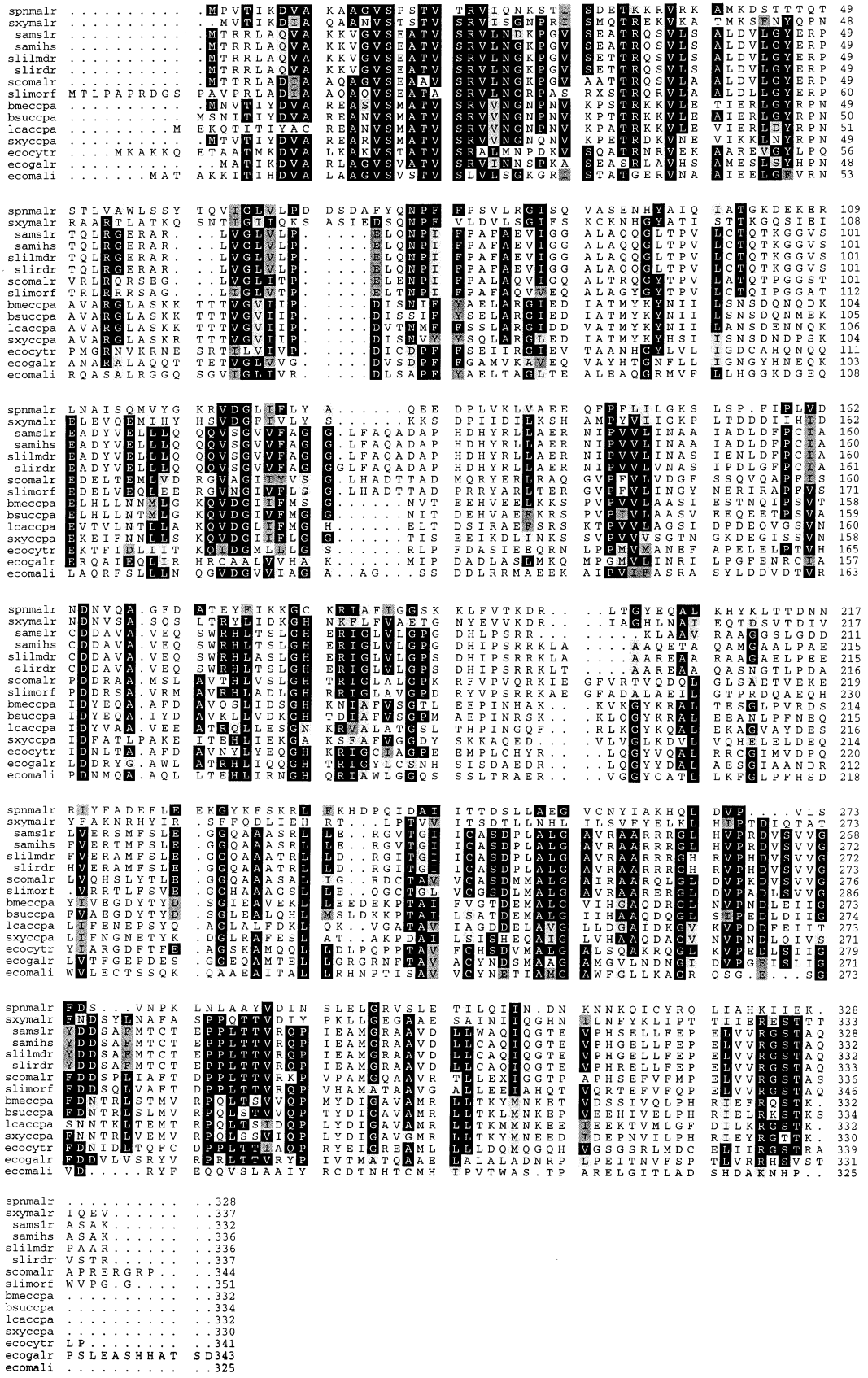
A



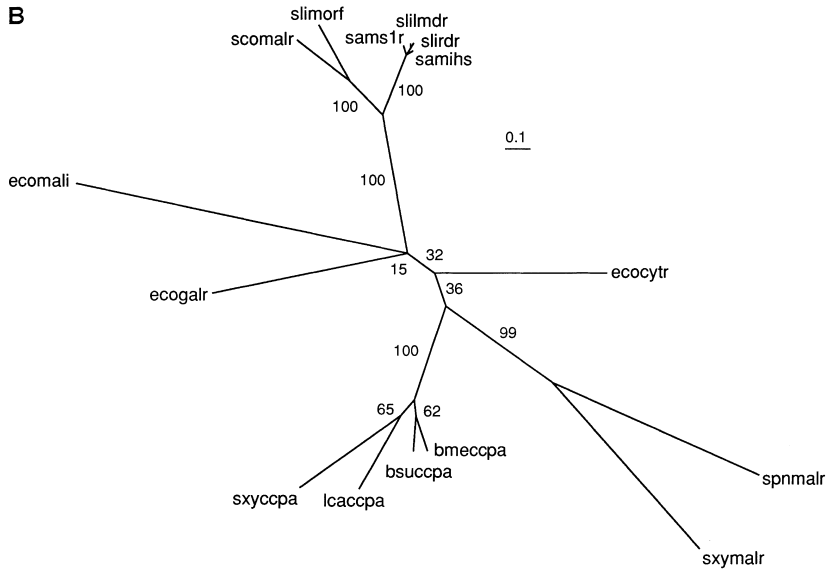


Fig. 1. A. Alignment of the amino acid sequences of a selection of MalR homologues: spnmalr, MalR of *Sta. pneumoniae* (SWISSPROT Q08511); sxymalr, MalR of *Sta. xylosus* (PIR S44187); samihs, product of the left direct repeat of AUD6 of *S. ambofaciens* (PIR S33360); sams1r, product of the right direct repeat of AUD6 of *S. ambofaciens* (PIR S33361); slilmdr, products of the left and middle direct repeats of AUD1 of *S. lividans* (PIR S21351/2); slirdr, product of the right direct repeat of AUD1 of *S. lividans* (PIR S21353); scomalr, MalR of *S. coelicolor* (this study; EMBL Accession no. Y07706); slimorf, product of ORF-*Sl* of *S. limosus* (this study; EMBL Accession no. Y08304); bmeccpa, CcpA of *B. megaterium* (EMBL L26052); bsuccpa, CcpA of *B. subtilis* (EMBL Accession no. M85182); lcaccpa, CcpA of *L. casei* (GenBank Accession no. M85182); sxyccpa, CcpA of *Sta. xylosus* (EMBL Accession no. X95439); ecocytr, CytR of *E. coli* (SWISSPROT P06964); ecogalr, GalR of *E. coli* (SWISSPROT P03024); ecomali, MalI of *E. coli* (SWISSPROT P18811).
B. Phylogenetic tree of the sequences aligned in (A). The number of supporting bootstrap replicates (out of 100) is shown for each internal branch.

as a pleiotropic effector of glucose repression (Hueck and Hillen, 1995; Henkin, 1996; Egeter and Brückner, 1996). In *S. coelicolor*, the most genetically characterized streptomycete, inactivation of an ATP-dependent glucose kinase encoded by *glkA* results in the inability to utilize glucose and in a pleiotropic loss of glucose repression (Hodgson, 1982; Seno and Chater, 1983; Kwakman and Postma, 1994), but has no effect on glucose transport (Hodgson, 1982). Moreover, replacement of GlkA with an unrelated glucose kinase from *Zymomonas mobilis*, or with a normally cryptic glucose kinase of *S. coelicolor*, conferred glucose utilization, but not glucose repression (Angell *et al*., 1994). Thus, *glkA* plays a key regulatory role in mediating glucose repression in *S. coelicolor*, and its homologue in *Sta. xylosus* appears to have a similar function (Wagner *et al*., 1995).

Earlier studies of the *aml* genes of *S. limosus* (Long *et al*., 1987) and *S. venezuelae* (Virolle *et al*., 1988) identified the 3′ end of a gene located immediately upstream of *aml* in both strains that appeared to encode a member of the LacI–GalR family of regulatory proteins. In this study we report the sequence of this upstream gene from *S. limosus*, and its use to isolate and characterize a homologue from *S. coelicolor*, *malR*, that is required for both substrate induction and glucose repression of maltose utilization.

Results

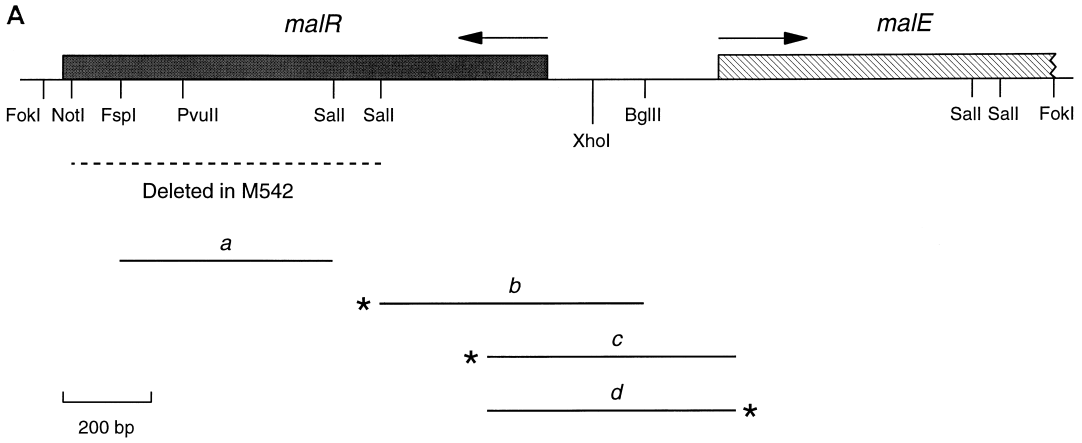
Isolation and sequence analysis of members of the lacI–galR *family of regulatory genes from* S. limosus *and* S. coelicolor

Sequence analysis of the *aml* genes of *S. limosus* and *S. venezuelae* revealed the 3′ end of an upstream open reading frame (ORF) whose predicted product showed significant amino acid sequence identity to the LacI–GalR family of regulatory proteins. The sequence (EMBL, Accession no. Y08304) of a 2.3 kb region upstream of *aml* of *S. limosus* was determined and revealed a complete ORF (ORF-*Sl*) that would encode a protein of 351 amino acids (39 kDa) with a significant degree of sequence identity to members of the LacI–GalR family (e.g. 29% and 30% amino acid sequence identity to LacI and GalR, respectively; Fig. 1A). Upstream of ORF-*Sl* lies the 3′ end of an ORF (*aglA*) whose predicted product is a homologue (57% identity over 330 amino acids) of an α-glucosidase from the actinomycete *Thermomonospora curvata* (EMBL, Accession no. U17917). *aglA* and ORF-*Sl* are co-transcribed (J. White, unpublished results) and apparently translationally coupled, with the TGA stop codon of *aglA* overlapping the ATG start codon of ORF-*Sl*.

As *S. limosus* is not a genetically well-characterized and

manipulable strain, further attempts to analyse the role of ORF-*Sl* were carried out by isolating and studying its homologue in *S. coelicolor*. To obtain the ORF-*Sl* homologue of *S. coelicolor*, a 32-fold degenerate oligonucleotide corresponding to the C-terminal segment of the helix-turn-helix motif of ORF-*Sl* (see below) was used to screen the ordered *S. coelicolor* cosmid library (Redenbach *et al*., 1996) by colony hybridization. DNA was isolated from 16 positive clones, digested with *Bam*HI and *Sal*I, and subjected to Southern analysis using the 1300 bp *Aat*II fragment (EMBL, Accession no. Y08304) containing most of ORF-*Sl* and part of *aglA* as probe. One cosmid (10B7) gave a strong hybridization signal, which was subsequently localized to a 13 kb *Bam*HI fragment. This fragment was cloned in the *Bam*HI site of pBR329, yielding pIJ2564. Double-strand sequencing of this plasmid using the oligonucleotide used to probe the ordered cosmid library revealed a close homologue of ORF-*Sl*. Sequencing of appropriate subcloned fragments from pIJ2564 revealed an ORF (*malR*) that would encode a protein of 344 aa (39 kDa). The cosmid maps at approx. 11 o'clock, on *Ase*I fragment C, of the combined physical and genetic map of the *S. coelicolor* chromosome (Redenbach *et al*., 1996).

A restriction map of the 2.2 kb *Fok*I fragment containing *malR* is shown in Fig. 2A. Upstream of and in the opposite orientation to *malR* lies a gene (*malE*) encoding a homologue of the maltose-binding protein found in other bacteria (Duplay *et al*., 1984; Puyet and Espinosa, 1993).



B

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-519  ACGTCGAGGGCGGCGAGCACGGACTGGCGGGTGGTGGCGGCGACGCCCGGCTTCCCGTTG
      TGCAGCTCCCGCCGCTCGTGCCTGACCGCCCACCACCGCCGCTGCGGGCCGAAGGGCAAC
       V  D  L  A  A  L  V  S  Q  R  T  T  A  A  V  G  P  K  G  N

           mal01
-459  AGGACGCGGCTGACGGTCGCTTCGCTCACCCCCGCCTGGGCGGCGATGTCGGCAAGCCGT
      TCCTGCGCCGACTGCCAGCGAAGCGAGTGGGGGCGGACCCGCCGCTACAGCCGTTCGGCA
       L  V  R  S  V  A  A  E  S  V  G  A  Q  A  A  I  D  A  L  R

-399  GTGGTCACAGGGGTGGACTGTACCTGTCGGCCGCTCCCTTGCACACCGATCGGGCGCCGT
      CACCAGTGTCCCCACCTGACATGGACAGCCGGCGAGGGAACGTGTGGCTAGCCCGCGGCA
       T  T fM          -10                    -35
          MalR

-339  GGGCGGCCTGTTGGACGGCCCGTCGGACGGCCCGGCGCGGGCTGCTGCGTCATCGCGCTC
      CCCGCCGGACAACCTGCCGGGCAGCCTGCCGGGCCGCGCCCGACGACGCAGTAGCGCGAG

-279  CCTCGAGAAAGTGGCGGCAAGAGCTTGCAGAGTCTTGCACAACCCGCCCGATACCGCTCG
      GGAGCTCTTTCACCGCCGTTCTCGAACGTCTCAGAACGTGTTGGGCGGGCTATGGCGAGC

-219  GGCCCCGTAAACAAGCGTCTCACGACGGTCGCCGAGAGGTCACGCCCGGATAACTTCGGA
      CCGGGGCATTTGTTCGCAGAGTGCTGCCAGCGGCTCTCCAGTGCGGGCCTATTGAAGCCT

                                          -35                -10
-159  GATCTCTTGCACATTTTTGCAGCAAGACCTTTCGCCAGGCTTTCAACGCTGTTACGTTCA
      CTAGAGAACGTGTAAAAACGTCGTTCTGGAAAGCGGTCCGAAAGTTGCGACAATGCAAGT

 -99  CGTCGCCCGACGGCAGCAACGAAGCGGTCGGCGGTCGGCAGGAGCGGCGGACGGGCCCGC
      GCAGCGGGCTGCCGTCGTTGCTTCGCCAGCCGCCAGCCGTCCTCGCCGCCTGCCCGGGCG

                                          MalE
                                    SD    fM R  R  G  I  A  A
 -39  ACCCTGCACCACACGGGCTTTCACCCTCAAGGAGAACTCATGCGGCGTGGCATAGCGGCC
      TGGGACGTGGTGTGCCCGAAAGTGGGAGTTCCTCTTGAGTACGCCGCACCGTATCGCCGG
                                                      mal02

       T  A  L  V  A  S  L  A  L  A  A  T  A  C  G  G  D  G  D  S
 22   ACCGCGCTGGTGGCGTCCCTCGCCCTGGCGGCGACGGCGTGCGGCGGAGACGGCGACAGC
      TGGCGCGACCACCGCAGGGAGCGGGACCGCCGCTGCCGCACGCCGCCTCTGCCGCTGTCG
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Fig. 2. A. Restriction map of *malR* and the 5′ region of *malE* of *S. coelicolor*. Arrows indicate the direction of transcription. a, *Fsp*I–*Sal*I fragment (477 bp) used for Southern hybridization and creation of the *malR* insertion mutant M541; b, *Sal*I–*Bgl*II fragment (564 bp) used for Southern hybridization, and S1 nuclease mapping and *in vitro* transcription analysis of *malR* transcripts; c and d, PCR fragments (549 bp) used for S1 nuclease mapping and *in vitro* transcription assays of *malR* and *malE* transcripts, respectively. Asterisks indicate ^{32}P-labelled ends. The extent of the in-frame deletion in *malR* is shown as a dotted line below the restriction map.

B. Nucleotide sequence of the intergenic region between *malR* and *malE*. The deduced amino acid sequences of MalR and MalE are shown below and above the nucleotide sequence, respectively. The *malR* transcription start site is shown in bold at position −392; putative −35 and −10 regions and a *malE* Shine–Dalgarno sequence (SD), presumably involved in ribosome binding, are indicated by lines above the nucleotide sequence. Direct and inverted repeats are indicated by arrows. The sequences of the oligonucleotides mal01 and mal02 used in the PCR and for sequencing are underlined.

Disruption of *malE* prevented the utilization of maltose as carbon source (G. P. van Wezel, J. White, M. J. Bibb and P. O. Postma, submitted). The sequence of the *malR–malE* intergenic region and the predicted translation start sites for *malR* and *malE* are shown in Fig. 2B.

Relationship of MalR to other members of the LacI–GalR family

In addition to ORF-*Sl*, the complete nucleotide sequences of five other streptomycete homologues of *malR* are available. Three occur as part of the amplifiable element AUD1 of *S. lividans* (Piendl *et al*., 1994), where they appear to play a role in DNA amplification (Volff *et al*., 1996), and two occur as part of the amplifiable element AUD6 of *Streptomyces ambofaciens* (Aubert *et al*., 1993); pairwise comparisons between the products of the six ORFs reveal 92–100% amino acid sequence identity (two of the homologues in AUD1 are identical). MalR and the product of ORF-*Sl* show a higher level of similarity to each other (63% identity) than to the products of the *S. ambofaciens* and *S. lividans* ORFs (53–55% identity). An alignment of the *Streptomyces* MalR homologues with members of the LacI–GalR family of proteins from other bacteria is shown in Fig. 1A, and a phylogenetic tree is shown in Fig. 1B. The *Streptomyces* homologues all group closely together, with MalR and the product of ORF-*Sl* forming a separate branch from the *S. lividans* and *S. ambofaciens* homologues. Both the protein sequence comparison and the phylogenetic tree indicate that MalR and the product of ORF-*Sl* are not significantly more similar to specific regulatory proteins for maltose utilization (e.g. MalR from *Streptococcus pneumoniae* (Puyet *et al*., 1993) and *Sta. xylosus* (Egeter and Brückner, 1995), and MalI from *E. coli* (Reidl *et al*., 1989)) than they are to the pleiotropic regulatory proteins CcpA (from *B. subtilis* (Henkin *et al*., 1991), *B. megaterium* (Hueck *et al*., 1994), *Sta. xylosus* (Egeter and Brückner, 1996) and *Lactobacillus casei* (GenBank, Accession no. U28137), and CytR (from *E. coli*; Valentin-Hansen *et al*., 1986). A feature shared by all members of this family of repressor proteins is a well-conserved N-terminally located helix-turn-helix motif responsible for DNA binding (Weickert and Adhya, 1992); such a motif is also present in the products of *malR* (Fig. 1A, scomalr, amino acid residues 5–24) and ORF-*Sl* (Fig. 1A, slimorf, amino acid residues 16–35).

The malR *transcript lacks an untranslated leader sequence*

To determine the transcription start site of *malR*, RNA was isolated from *S. coelicolor* M145 grown in liquid minimal medium (SMM) containing glucose as carbon source and subjected to S1 mapping. A single transcription start site was located corresponding to the first G of the predicted GTG translation start codon (Fig. 3). Thus, the *malR* transcript appears to lack a conventional ribosome-binding site, a property shown by several other streptomycete mRNAs (Janssen, 1993; Strohl, 1992). *In vitro* transcription assays using *S. coelicolor* RNA polymerase with a 564 bp *Sal*I–*Bgl*II fragment and a 549 bp polymerase chain reaction (PCR) product (Fig. 2A; fragments b and c, respectively), each containing the *malR* promoter region as templates, gave the expected run-off transcripts of ≈330 nucleotides (nt) and 120 nt, respectively. The transcriptional start site of *malR* is preceeded by sequences (Fig. 2B, 5′-TGTGCA–17 bp–TACAGT-3′) that are similar to the proposed consensus sequence (5′-TTGACN–16–18 bp–TAGAPuT-3′; Strohl, 1992) for promoters recognized by the major RNA polymerase holoenzyme of *Streptomyces*.

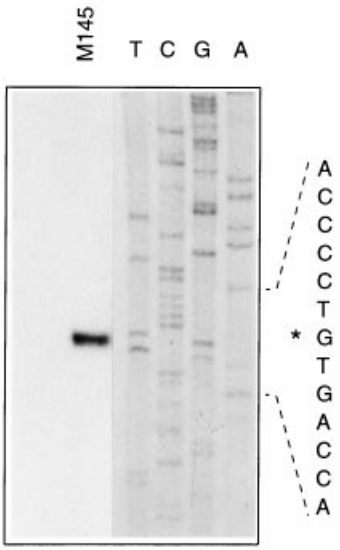

Fig. 3. Determination of the transcription start site of *malR*. TCGA, *malR* nucleotide sequence ladder. M145, RNA-protected fragment derived from RNA isolated from M145 grown in SMM containing glucose as carbon source; the asterisk indicates the most probable transcription start site, and coincides with the first G of the predicted translation start codon GTG.

S1 nuclease protection studies using RNA isolated from M145 grown in SMM containing glucose revealed *malR* transcripts throughout growth, but with maximal levels during mid- and late-exponential phases (Fig. 4).

Inactivation of malR *causes constitutive, enhanced and glucose-insensitive transcription of* malE

To determine the function of *malR* and its possible role in the regulation of *malE*, an in-frame deletion was made that removed the C-terminal two-thirds of the *malR*-coding region (corresponding to amino acid residues 112–341

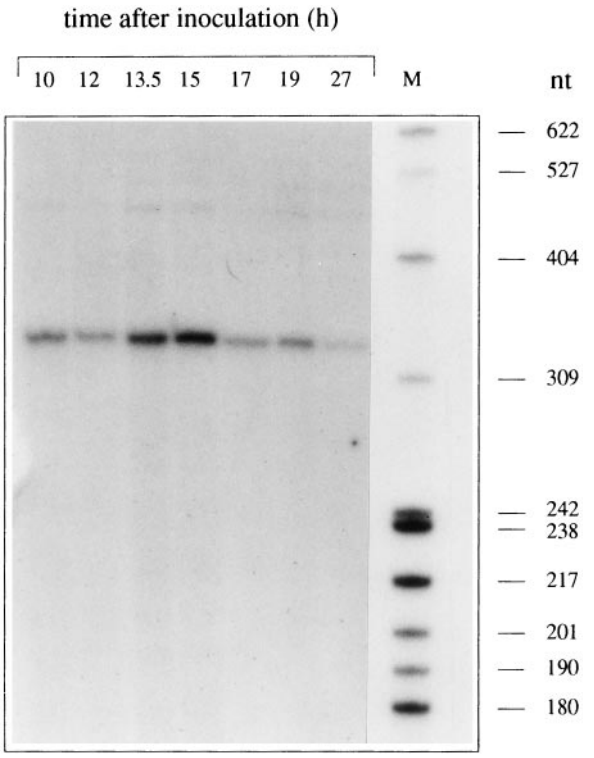

Fig. 4. Transcription of *malR* during growth in SMM containing glucose as carbon source. M, ^{32}P-end-labelled *Hpa*II-digested pBR322 size markers; nt, nucleotides. The culture entered stationary phase after ≈22 h.

out of 344). Such a mutation should not have a polar effect on the expression of genes 3′ of *malR*. *S. coelicolor* J1501 was transformed with single-stranded DNA derived from pIJ2591 (Fig. 5), which carries *tsr* conferring thiostrepton-resistance (Thio^R). One Thio^R isolate, which had probably arisen by single cross-over integration of pIJ2591 into the *malR* region of the chromosome, was subjected to three rounds of sporulation on non-selective SFM agar plates to allow a second cross-over to occur, resulting in loss of *tsr*. DNA was isolated from 40 Thio^S colonies, digested with *Not*I and analysed in two Southern blots. In the first hybridization, using the 564 bp *Sal*I–*Bgl*II fragment corresponding to the N-terminal part of *malR* as probe (Fig. 2A, fragment b), the 2.2 kb *Not*I fragment containing *malR* should have been 700 bp smaller in the deletion mutant, while in the second hybridization, using the 477 bp *Fsp*I–*Sal*I probe (Fig. 2A, fragment a) recognizing the part of *malR* that should have been deleted, no hybridization signal was expected in the mutant. One of the Thio^S clones (M542) gave the correct patterns. A second *malR* mutant (M541) was made by integrating pIJ2587 containing the 477 bp *Fsp*I–*Sal*I fragment internal to *malR* (Fig. 2A, fragment a) in the chromosome of M145; disruption of *malR* was confirmed by Southern analysis.

In agreement with data obtained for other *S. coelicolor* strains (Hodgson, 1980), maltose proved to be a poor carbon source for *S. coelicolor* M145 and J1501, giving growth rates and final biomass accumulations that were consistently lower than those obtained with glucose (data for M145 are shown in Fig. 6A). In contrast, the growth rates of the *malR* mutants M541 and M542 on maltose were comparable to those for glucose-grown cultures and significantly higher than the congenic *malR*$^+$ parental strains (data for M542 are shown in Fig. 6B). Furthermore, the final biomass accumulation of M541 and M542 grown on maltose approached that obtained with glucose (Fig. 6B), indicating an increased ability to use maltose as carbon source.

To assess whether this might reflect derepression of *malEFG* and elevated levels of maltose uptake, transcription of *malE* in M145 and in M542 was analysed by S1 nuclease protection assays using RNA isolated from liquid minimal medium (NMMP) cultures containing maltose, glucose or a combination of maltose and glucose (earlier studies had shown that transcription of *malE* was barely detectable when mannitol was used as a non-repressing carbon source, but markedly induced on addition of maltose (G. P. van Wezel, J. White, M. J. Bibb and P. O. Postma, submitted)). In M145, transcription of *malE* was strongly induced by maltose, and this induction was completely repressed when glucose was present as an additional carbon source (Fig. 7). However, in M542 transcription of *malE* was constitutive and occurred at levels much greater than the induced level in M145. Moreover, transcription of *malE* was no longer repressed by

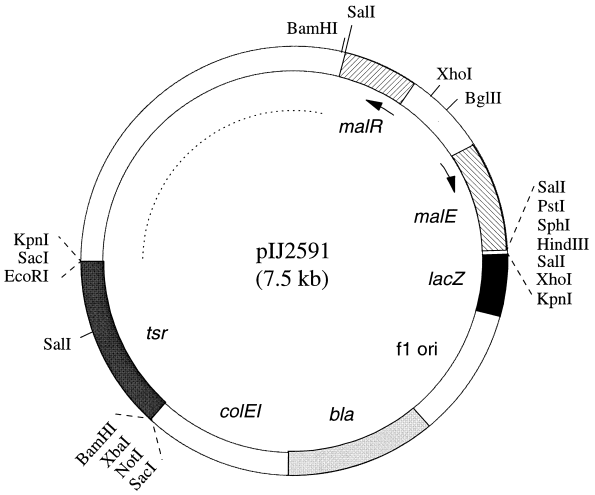

Fig. 5. Restriction map of pIJ2591. ColE1 and f1 ori, origins of replication; *bla*, ampicillin-resistance gene; *tsr*, thiostrepton-resistance gene; *lacZ*, segment containing the *lacZ* promoter and encoding the α fragment of β-galactosidase. The dotted line denotes sequences located 3′ of *malR*, and arrows indicate the direction of transcription.

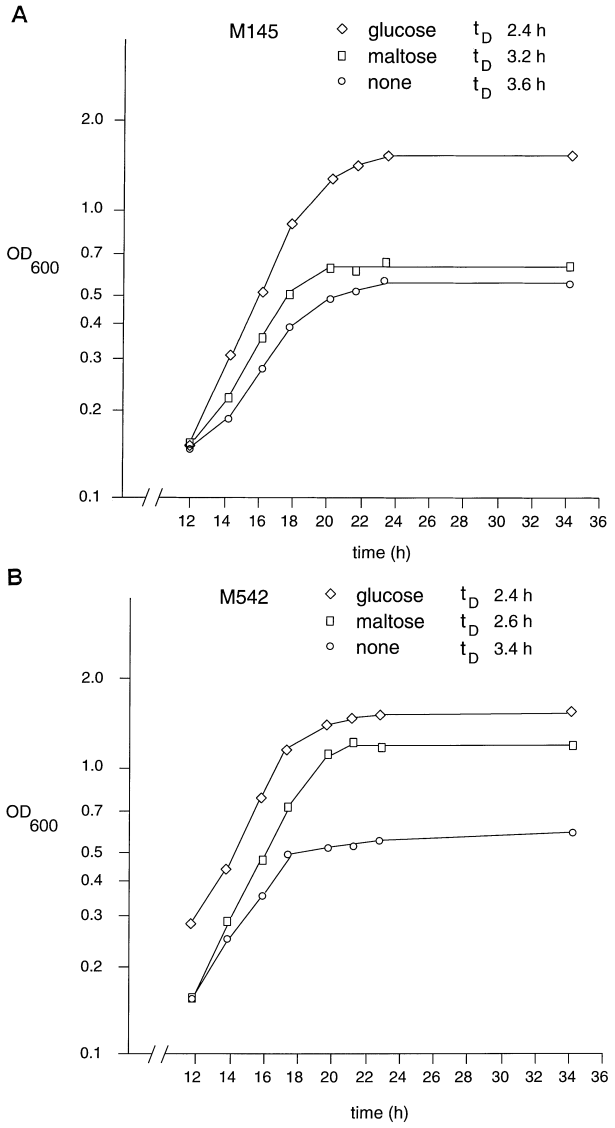

Fig. 6. Growth of *S. coelicolor* M145 (*malR*$^+$) (A) and *S. coelicolor* M542 (Δ*malR*) (B) in the liquid minimal medium SMM containing 0.1% (w/v) casamino acids supplemented with 1% (w/v) glucose, maltose or no additional carbon source. t_D, doubling time.

glucose, indicating a role for MalR in both substrate induction and catabolite repression of maltose utilization. Similar results were obtained with M541.

To confirm that the enhanced ability of M542 to utilize maltose was due solely to deletion of *malR*, the 2.2 kb *Fok*I fragment containing the *malR*-coding region and promoter (Fig. 2A) was cloned in the conjugative vector pSET152 yielding pIJ2593, which was subsequently integrated at the chromosomal øC31-attachment site, resulting in M543. The growth rate and biomass accumulation of M543 in SMM containing maltose were essentially the same as those of M145, confirming restoration of the wild-type phenotype.

Inactivation of malR *represses agarase production*

Expression of the agarase gene (*dagA*) of *S. coelicolor* is subject to carbon catabolite repression (Hodgson, 1980; Bibb *et al*., 1987; Kwakman and Postma, 1994), and *dagA* transcription is strongly repressed by glucose (Servín-González *et al*., 1994; Angell *et al*., 1994). Agarase production by M145, the *glkA* mutant J1915, and the *malR* mutants M541 and M542, was readily detected on MM plates containing agar as sole carbon source (Fig. 8; upper left plate); as expected, agarase production by all except J1915 was effectively repressed by glucose (Fig. 8; upper right plate). Unexpectedly, while agarase production by M145 and J1915 was readily detected on plates containing 1% (w/v) maltose, agarase production by M541 and M542 was undetectable (Fig. 8; bottom left plate). This apparent repression of agarase activity in the *malR* mutants might reflect higher levels of intracellular glucose which could arise from elevated levels of maltose uptake upon the observed derepression of *malEFG* (Fig. 7). Agarase production in glucose-grown agar cultures of M543 was restored, confirming that repression did indeed result from inactivation of *malR*.

Overexpression of malR *prevents maltose utilization*

The 2.2 kb *Fok*I fragment containing *malR* was cloned in the multicopy vector pIJ486 (*c*. 50–100 copies per genome), yielding pIJ2592. Introduction of pIJ2592 into M145 prevented growth in liquid minimal medium (NMMP lacking casamino acids) containing maltose as sole carbon source, presumably because of overexpression of

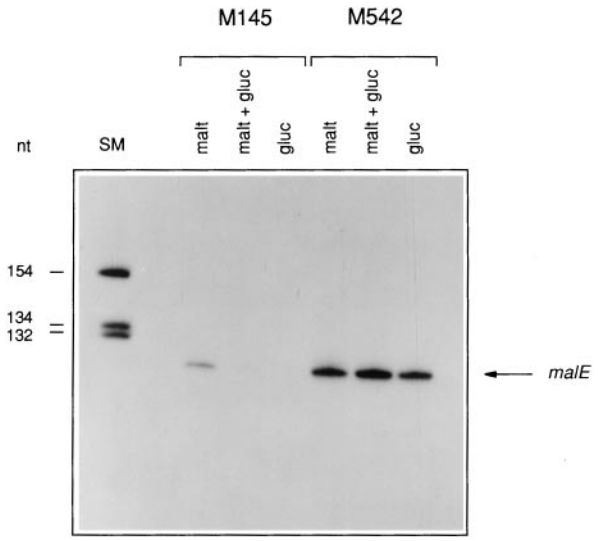

Fig. 7. Transcription of *malE* in SMM-grown cultures of M145 and M542, using maltose (malt), maltose plus glucose (malt + gluc) or glucose (gluc) as carbon sources. SM, ^{32}P-end-labelled *Hpa*II-digested pBR322 size markers; nt, nucleotides.

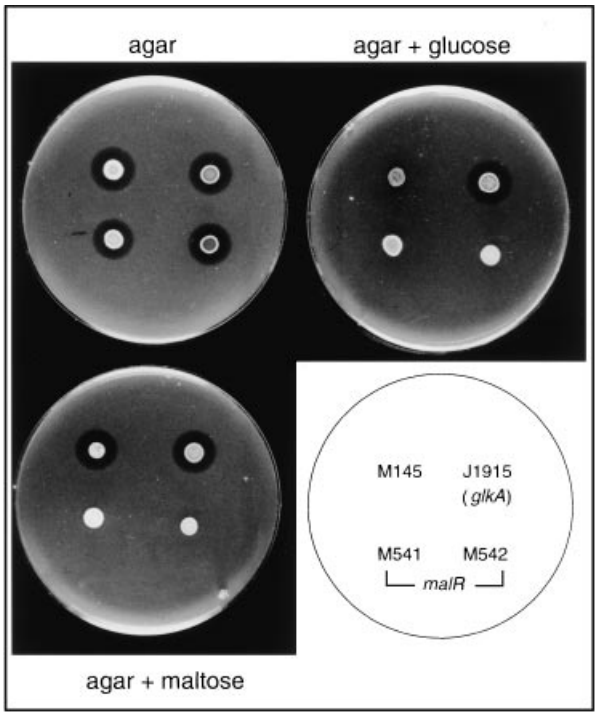

Fig. 8. Effect of carbon source on agarase production by M145, J1915 (M145 *glkA*), and the *malR* mutants M541 and M542. A volume of 4 μl of a titred spore preparation of each of the four strains was spotted on MM plates, and agarase activity detected as zones of clearing of the agar.

malR and enhanced repression of *malE*. No growth inhibition was detected when glucose, glycerol, mannitol, arabinose or galactose were used as carbon sources.

Identification of MalR using antibodies raised against CcpA from B. megaterium

As MalR and CcpA from *B. megaterium* are 30% identical (Fig. 1A), we assessed whether antibodies raised against CcpA would cross-react with MalR. S30 supernatants derived from total-protein extracts prepared from 36 h TSB-grown cultures of *S. coelicolor* were analysed by Western blotting using antibodies raised against CcpA of *B. megaterium* (Küster *et al*., 1996). Two proteins of approx. 55 kDa and 43 kDa were detected in extracts from M145; bands of a similar mobility were noted in extracts of *S. coelicolor* DSM 40233 by Küster *et al*. (1996). The 43 kDa band is approx. the same size as that predicted for MalR (39 kDa). It was not observed in extracts from the *malR* deletion mutant M542, but reappeared in an extract from M542 containing pIJ2592 at a level several times higher than in M145. We therefore believe that the 43 kDa protein is MalR. The 55 kDa protein (≈500 amino acids) is significantly larger than any known member of the LacI–GalR family of proteins.

Discussion

Members of the *lacI–galR* family of regulatory genes were identified in *S. limosus* (ORF-*SI*) and *S. coelicolor* (*malR*), located upstream of *aml* (encoding an α-amylase; Long *et al*., 1987) and *malE* (probably encoding a maltose-binding protein; G. P. van Wezel, J. White, M. J. Bibb and P. O. Postma, submitted), respectively. The degree of similarity of the two gene products (63% amino acid sequence identity) is consistent with the notion that they are functionally homologous proteins. As attempts at phage-mediated disruption of ORF-*SI* in *S. limosus* failed (J. White, unpublished results), we focussed on its homologue from the genetically more amenable *S. coelicolor*.

Transcription of *malE* (and probably of *malFG*, both of which appear to be required for maltose uptake; G. P. van Wezel, J. White, M. J. Bibb and P. O. Postma, submitted) was induced by maltose in *S. coelicolor* M145 (G. P. van Wezel, J. White, M. J. Bibb and P. O. Postma, submitted; this study), but was constitutive in the *malR* mutants M541 and M542. Presumably, in the wild-type strain, maltose or a maltose metabolite binds to MalR and prevents it from repressing transcription initiation at the *malE* promoter. Disruption or deletion of *malR* also relieved glucose repression of *malE* transcription. Thus, MalR is required for both substrate induction and glucose repression of *malE* expression. While *S. coelicolor* grows poorly on maltose as sole carbon source (Hodgson, 1982; G. P. van Wezel, J. White, M. J. Bibb and P. O. Postma, submitted), the *malR* mutants grew much better than the parental strain, perhaps because of elevated levels of maltose uptake. Consistent with this, the level of *malE* transcription in the *malR* mutant M542 far exceeded the induced level observed in M145 on all carbon sources tested. Furthermore, in contrast to M145, agarase production by the *malR* mutants was repressed by maltose, perhaps reflecting higher levels of intracellular glucose, a potential consequence of improved maltose uptake. While compatible with its role as a repressor, the ability of 50–100 copies of *malR* (in the form of pIJ2592) to prevent growth of *S. coelicolor* M145 on maltose is surprising. This may reflect the ability of enhanced levels of MalR to completely repress *malE* transcription, preventing inducer uptake.

Notwithstanding the ability of antibodies raised against CcpA of *B. megaterium* to cross-react with MalR, disruption of *malR* had no apparent pleiotropic effect on carbon source utilization, and we have no evidence to suggest that MalR is a functional homologue of CcpA. Homologues of MalR occur in *Sta. pneumoniae* (Puyet *et al*., 1993) and in *Sta. xylosus* (Egeter and Brückner, 1995). While MalR of *S. coelicolor* and *Sta. pneumoniae* acts to repress maltosaccharide and maltose utilization, respectively, inactivation of *malR* in *Sta. xylosus* reduces the level of maltose

transport and utilization. The role that these latter two homologues play in glucose repression of maltosaccharide and maltose catabolism in their respective hosts was not reported.

The ability of *malR* to mediate both substrate induction and catabolite repression of *malE* transcription resembles the role of the phylogenetically unrelated GylR, the repressor of the glycerol operon *gylCABX* of *S. coelicolor* (Hindle and Smith, 1994). The level of *gylCABX* transcription in *gylR* null mutants is markedly increased in both uninduced and glucose-grown cultures, and the presence of *gylR* on a multicopy plasmid prevents glycerol utilization (F. Amini, M. S. B. Paget and C. P. Smith, personal communication). Whether the dual roles that GylR and MalR play in the regulation of catabolic pathways in *S. coelicolor* is a common occurrence in streptomycetes remains to be determined.

The existence of a regulatory protein that mediates both substrate induction and glucose repression of *malE* transcription predicts the existence of *cis*-acting sequences that are required for both aspects of regulation. Direct and inverted repeats that might fulfil such a function occur 5′ and 3′ of the *malE* promoter (Fig. 2B). The 5′ direct repeat CTTGCA and the inverted repeat 5′-TCTTGC–11 bp–GCAAGA-3′, which occurs just upstream of the putative −35 region of *malE*, are also found in the promoter region of *aml* of *S. limosus*. The direct repeat appears to play a role in the induction of *aml* transcription by maltose, and deletion of the inverted repeat resulted in constitutive expression that was insensitive to glucose repression (Virolle and Gagnat, 1994). Consequently, this inverted repeat is a probable binding site for MalR. Direct repeats that show no sequence similarity to the direct and inverted repeats present in the *malE* promoter region are also required for both substrate induction and glucose repression of a chitinase gene (*chi63*) of *Streptomyces plicatus* (Delić *et al*., 1992), again consistent with the notion of a single regulatory protein with dual functions in chitinase gene regulation.

The role of a repressor in both induction and glucose repression of *malE* transcription could be readily explained if glucose repression was mediated by inducer exclusion, i.e. if glucose, directly or indirectly, prevented the uptake of maltose. Interestingly, glucose repression of lactose utilization during growth of *E. coli* on both sugars appears to be totally attributable to inducer exclusion and to the level of active LacI, the repressor of the *lac* operon, with cAMP and CRP (cyclic AMP receptor protein) playing no role (Inada *et al*., 1996). Although there is no evidence either for or against the role of inducer exclusion in regulating *malE* expression, constitutive expression of *aml* of *S. limosus* in *S. lividans*, achieved by cloning the gene on a high-copy-number plasmid, was still subject to glucose repression (Virolle and Bibb, 1988), i.e. under conditions where *aml* transcription was inducer independent, glucose repression was still operative. If applicable to the closely related *S. coelicolor*, and to other genes in the maltose regulon, this suggests that inducer exclusion does not play a major role in glucose repression of *malE* transcription (similar conclusions were drawn for glucose repression of *dagA* expression in *S. coelicolor* (Servín-González *et al*., 1994)). How else might glucose repression of *malE* transcription be mediated in a MalR-dependent manner? There is evidence in *Bacillus* species that glucose-6-phosphate acts as an anti-inducer of XylR, the repressor of the xylose-utilization operon, both *in vivo* and *in vitro*, by competing with xylose for binding to XylR (Scheler and Hillen, 1993; Dahl *et al*., 1995); although inactivation of *xylR* reduces glucose repression only about twofold in *B. megaterium* (Schmiedel and Hillen, 1996), much less than the apparent effect of deleting *malR* on glucose repression of *malE* transcription (Fig. 7), it is possible that a similar mechanism operates in *S. coelicolor*. Alternatively, perhaps an unidentified pleiotropic regulatory protein responsible for glucose repression, and functionally analagous to CcpA, requires the presence of MalR to bind to the *malE* promoter region. Finally, as the glucose kinase gene (*glkA*) of *S. coelicolor* plays a pleiotropic role in carbon catabolite repression, and because GlkA is required for glucose repression of the *aml* genes of *S. limosus* and *S. venezuelae* when cloned in *S. coelicolor* (Virolle and Bibb, 1988; Virolle *et al*., 1988), glucose repression of *malE* may be mediated by MalR through interaction with, or modification by, GlkA.

malR is transcribed constitutively during growth of *S. coelicolor* in liquid culture, with transcript levels peaking during mid- to late-exponential phase. The transcription start site of the *malR* promoter coincides with the first G of the predicted GTG translational start codon, and thus the *malR* transcript lacks a conventional untranslated leader sequence and ribosome-binding site, consistent with the absence of a purine-rich Shine–Dalgarno sequence complementary to the 3′ end of the 16S RNA upstream of the *malR*-coding region. Although several streptomycete mRNAs lack untranslated leader sequences (Janssen, 1993; Strohl, 1992), the *malR* transcript appears to be only the second example of a leaderless mRNA involved in primary metabolism, the other being that derived from the histidase gene of *Streptomyces griseus* (Wu *et al*., 1995).

Experimental procedures

Bacterial strains, culture conditions, plasmids and phages

E. coli K-12 strains JM101 and JM109 (Messing *et al*., 1981), and ET12567 mini-F′Km (MacNeil *et al*., 1992; M. J. Bibb, unpublished) were used for routine subcloning and for the preparation of single-stranded DNA, respectively, and were

grown and transformed by standard procedures (Sambrook *et al*., 1989); transformants were selected with carbenicillin at a final concentration of 200 μg ml^{-1}. Luria (L) broth containing 50 μg ml^{-1} kanamycin was used to grow ET12567 mini-F′Km to isolate single-stranded DNA using M13KO7 as helper phage (Sambrook *et al*., 1989). *S. coelicolor* A3(2) strains used were M145 (Hopwood *et al*., 1985), J1501 (Chater *et al*., 1982) and J1915 (Kelemen *et al*., 1995). Protoplast preparation and transformation were as described by Hopwood *et al*. (1985). SFM medium (mannitol, 20 g l^{-1}; soya flour, 20 g l^{-1}; agar, 20 g l^{-1}, dissolved in tap water and autoclaved twice) is a modified version of that reported by Hobbs *et al*. (1989) and was used to make spore suspensions. Liquid minimal medium (NMMP; Hopwood *et al*., 1985) containing 1% (w/v) glucose, mannitol, maltose or maltose plus glucose, and unless otherwise stated 0.05% (w/v) casamino acids, or SMM (Strauch *et al*., 1992) containing 0.1% (w/v) casamino acids and 1% (w/v) glucose or maltose, were used to assess carbon-source utilization and for RNA isolation. MM plates (Hopwood *et al*., 1985) were used to assess agarase production, which was detected as zones of clearing of the agar. TSB (Oxoid Tryptone–Soya broth powder; 30 g l^{-1}) was used to grow *S. coelicolor* strains for Western analysis.

pUC18 (Yanisch-Perron *et al*., 1985), pBluescript-II SK+ (Stratagene), and pSET152 (Bierman *et al*., 1992) were used for cloning experiments. pIJ486 (Ward *et al*., 1986) was used as a high-copy-number vector (≈50–100 copies per chromosome; T. Kieser, personal communication) in *S. coelicolor*. The 2.2 kb *Fok*I fragment (Fig. 2A) containing *malR* and part of *malE* of *S. coelicolor* was cloned in pUC18, pIJ486 and pSET152, resulting in pIJ2588, pIJ2592, and pIJ2593, respectively. Standard procedures were used to isolate plasmid DNA from *E. coli* (Sambrook *et al*., 1989), and to isolate plasmid and total DNA from *S. coelicolor* (Hopwood *et al*., 1985).

pIJ2564 was made by cloning a 13 kb *Bam*HI fragment containing *malR* from cosmid 10B7 in pBR329 (Covarrubias and Bolivar, 1982). pIJ2587, which was used to make the *malR* disruption mutant M541, is a pUC18 derivative containing the internal 477 bp *Fsp*I–*Sal*I fragment of *malR* (Fig. 2A, fragment a) and *tsr*. pIJ2591 (Fig. 5), which was used to make the *malR* deletion mutant M542, is a pBluescript-II SK+ derivative containing *tsr* and a 3.5 kb segment from which the internal *Sal*I–*Not*I region of *malR* (Fig. 2A) had been deleted. Double- and single-stranded DNAs derived from pIJ2587 and pIJ2591, respectively, were used to transform protoplasts of *S. coelicolor* M145 and J1501, respectively, and integrants selected with a final concentration of 50 μg ml^{-1} Thio. Southern analyses were performed to confirm the mutations present in M541 and M542 using the appropriate ^{32}P-labelled probes (Sambrook *et al*., 1989) and previously described hybridization conditions (van Wezel *et al*., 1991).

DNA sequence analysis

The nucleotide sequence of *malR* was determined using the Promega TaqTrack and Pharmacia T7 sequencing kits and double-stranded DNA templates derived by subcloning DNA fragments from pIJ2588 and pIJ2564 in pUC18. For ORF-*Sl* and *aglA*, sonicated fragments of the 2.2 kb *Eco*RI–*Bcl*I chromosomal segment containing the genes (Virolle and Bibb, 1988) were cloned in the *Sma*I site of M13mp18, and nucleotide sequences determined using single-stranded DNA templates and the Klenow fragment of DNA polymerase I (Sanger *et al*., 1977). Synthetic oligonucleotides were used to close gaps in the sequences. The sequences of *S. coelicolor malR* (Accession no. Y07706) and *S. limosus* ORF-*Sl* with part of *aglA* (Accession no. Y08304) were deposited in the EMBL nucleotide sequence database.

Nuclease S1 protection assays

RNA was purified as described by Hopwood *et al*. (1985), except that DNase I treatment was used in addition to salt precipitation to eliminate DNA from the nucleic acid preparations. For each nuclease S1 protection assay, approx. 0.02 pmol (≈10^4 Cerenkov counts min^{-1}) of labelled probe was hybridized to 20 μg of RNA in Na-TCA buffer (Murray, 1986) at 45°C overnight after denaturation at 65°C for 15 min. All subsequent steps were carried out as described previously (Strauch *et al*., 1991), using an excess of probe. All of the nuclease S1 protection experiments were carried out at least twice using RNA isolated from independent cultures, and the results presented were shown to be reproducible. The probes used are shown in Fig. 2A. The 564 bp *Sal*I–*Bgl*II fragment of pIJ2588 (Fig. 2A, probe b), ^{32}P-end-labelled at the *Sal*I site, and the 549 bp polymerase chain reaction (PCR) product (Fig. 2A, probe c) made using oligonucleotides mal02 and ^{32}P-end-labelled mal01 (Fig. 2B), were used for mapping *malR* transcripts. The same PCR product, but made using unlabelled mal01 and ^{32}P-end-labelled mal02, was used for determining the level of *malE* transcripts. PCRs contained 1× PCR buffer (Boehringer Mannhein), 0.2 mM of each dNTP, 25–50 pmol of each primer, 10 ng of pIJ2564, 5 U Taq polymerase (Boehringer Mannhein) and 5% (v/v) glycerol in a total volume of 100 μl. Samples were subjected to 30 cycles of 60 s at 94°C, 60 s at 54°C and 60 s at 72°C.

In vitro *transcription analysis*

RNA polymerase was isolated from cultures in the transition phase between exponential growth and stationary phase, as described previously (Buttner and Brown, 1985). *In vitro* run-off transcription experiments were performed as described by Buttner *et al*. (1987) using the 564 bp *Sal*I–*Bgl*II fragment and the 549 bp PCR product (Fig. 2A, fragments b and c) as templates. Products were analysed on denaturing 6% (w/v) polyacrylamide gels using ^{32}P-end-labelled *Hpa*II fragments of pBR322 as size markers.

Western blots

Western analyses were conducted as described by Vijgenboom *et al*. (1994) using a 1:1000 dilution of antibodies raised against CcpA of *B. megaterium*.

Amino acid sequence analysis

The program CLUSTALW (Thompson *et al*., 1994) at the SEQNET facility (Daresbury Laboratory, Cheshire, UK) was

used to align the sequences, calculate distances using the Kimura correction, and construct the tree by the neighbour-joining method (Saitou and Nei, 1987) with 100 bootstrap replicates. TreeView (R. D. M. Page, University of Glasgow) was used to display the tree.

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